EXHIBIT E

Lipases and HDL metabolism

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Plasma levels of high-density lipoproteins (HDL) cholesterol are strongly inversely associated with atherosclerotic cardiovascular disease, and overexpression of HDL proteins, such as apolipoprotein A-I in animals, reduces progression and even induces regression of atherosclerosis. Therefore, HDL metabolism is recognized as a potential target for therapeutic intervention of atherosclerotic vascular diseases. The antiatherogenic properties of HDL include promotion of cellular cholesterol efflux and reverse cholesterol transport, as well as antioxidant, anti-inflammatory and anticoagulant properties. The molecular regulation of HDL metabolism is not fully understood, but it is influenced by several extracellular lipases. Here, we focus on new developments and insights into the role of secreted lipases on HDL metabolism and their relationship to atherosclerosis.

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HDL (see Glossary) play an important role in cholesterol homeostasis. They also protect the arterial wall from the development of atherosclerosis. One mechanism by which HDL protect is by promoting efflux of excess cholesterol from cells in the arterial wall, returning it to the liver for excretion into the bile, a process known as 'reverse cholesterol transport' [1,2]. However, there is evidence that HDL can protect LDL from oxidation [3], reduce the inflammatory response of endothelial cells [4,5], inhibit the coagulation pathway [6] and promote the availability of nitric oxide [7], so there might be other mechanisms by which HDL also protect against atherosclerosis.

HDL are macromolecules comprising lipids (phospholipids, cholesterol and some triglyceride), as well as apolipoproteins, the major one of which is apoA-I [8,9], which is synthesized and secreted by both the intestine and the liver. Nascent apoA-I-containing HDL particles interact with peripheral cells and acquire cholesterol and phospholipid through a transport process facilitated by the cellular protein ABCA1 (Fig. 1). Unesterified cholesterol is esterified to cholesteryl ester within the HDL particle by the enzyme LCAT. HDL cholesteryl ester can be taken up selectively by the liver through the action of the SR-BI. Cholesteryl ester can also be selectively transferred to apoB-containing lipoproteins in exchange for triglyceride through the action of CETP. Conversely, the PLTP mediates transfer of phospholipids from apoB-containing lipoproteins to HDL. Several members of the family of triglyceride lipase genes also influence the metabolism of HDL. Hydrolysis of triglycerides in triglyceride-rich lipoproteins by LPL results in transfer of lipids and apolipoproteins to HDL. HL hydrolyzes HDL triglyceride and phospholipids, generating smaller lipid-depleted HDL particles. Finally, EL might hydrolyze HDL phospholipids, thus promoting HDL catabolism. sPLA2 also has the ability to hydrolyze HDL phospholipids (Fig. 1). Here, we discuss the secreted lipases that are directly involved in HDL metabolism and detail the new developments in the field.

Lipoprotein lipase

LPL is synthesized in adipocytes and in both skeletal and cardiac myocytes. It is transported to the capillary endothelial surface where, bound to HSPGS, it hydrolyzes triglycerides in the triglyceride-rich lipoproteins, chylomicrons and VLDL [10]. Through its lipolytic action, LPL generates FFAS for conversion to triglyceride to be stored in adipocytes, and for energy use by skeletal and cardiac myocytes, and thus plays an important role in energy homeostasis [11]. After lipolysis of triglyceride-rich lipoproteins, surface phospholipids and apolipoproteins from these lipoproteins dissociate and are acquired by HDL. Indeed, transgenic overexpression of the gene encoding LPL in mice (*Lpl*) results in increased HDL cholesterol (HDL-C) levels [12]. Conversely, the gene knockout of *Lpl* is associated not only with severe hypertriglyceridemia, but also with very low HDL-C levels in targeted mice [13,14]. In *Lpl*-deficient mice rescued by crossbreeding with mice expressing LPL in cardiac muscle alone, plasma triglycerides and HDL-C levels were normalized in adult animals [15]. However, although mice expressing LPL only in skeletal muscle have normal triglycerides, they have reduced HDL-C levels compared with wild-type mice [16]. Therefore, the tissue source of the LPL might be an important determinant of its effects on HDL.

In humans, deficiency of LPL is also associated with severe hypertriglyceridemia and very low HDL-C levels [17]. Even heterozygosity for LPL deficiency in humans is associated with reduced HDL-C levels [18]. Post-heparin plasma LPL activity is directly correlated with plasma HDL-C levels [19]. Interestingly, the relatively common LPL variant Ser447Stop is associated with increased LPL activity and increased HDL-C levels [18,20].

The relationship between LPL expression and atherosclerosis is complex, and could depend on the cellular origin of the LPL. Transgenic overexpression of *Lpl* in multiple tissues, including muscle and adipose, was associated with increased HDL-C levels and reduced atherosclerosis in

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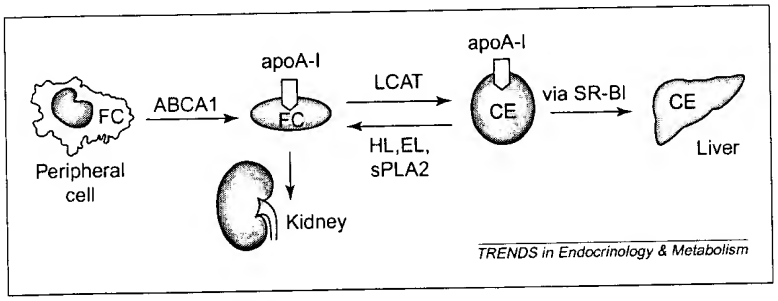

Fig. 1. A schematic diagram depicting the role of lipases in HDL metabolism. Lipid-poor apoA-I acquires free cholesterol (FC) from peripheral cells through an efflux process facilitated by the cellular protein ATP-binding cassette protein A1 (ABCA1). FC is converted to cholesteryl ester (CE) within the HDL particle through transfer of a fatty acid from phosphatidylcholine by the enzyme lecithin cholesterol acyltransferase (LCAT). HDL-CE can be taken up selectively by the liver through the action of the scavenger receptor class BI (SR-BI) and targeted for excretion in the bile. HDL-CE can also be selectively transferred to apoB-containing lipoproteins through the action of cholesteryl ester transfer protein and returned to the liver via the LDL receptor. Hepatic lipase (HL) hydrolyzes HDL triglyceride and phospholipid, remodeling larger HDL particles to smaller HDL particles, which are then at greater risk of catabolism via the kidney. Endothelial lipase (EL) and secretory phospholipase A2 (sPLA2) might also participate in the remodeling of HDL from larger to smaller particles. Lipoprotein lipase (LPL) is not shown but contributes to HDL formation by generating redundant phospholipids and apolipoproteins on apoB-containing lipoproteins that are transferred to HDL. Abbreviations: see Glossary.

Ldlr-deficient mice [21], *Apoe*-deficient mice [22] and cholesterol-fed rabbits [23]. Pharmacological upregulation of *Lpl* expression in rats was found to raise HDL-C levels and reduce atherosclerosis [24]. However, macrophage-derived *Lpl* was shown through bone marrow transplant studies to promote atherosclerosis without effects on HDL-C levels in mice [25], suggesting that the source of LPL expression could influence its effects on atherosclerosis. Overall, LPL derived from adipose tissue and muscle appears to increase HDL-C levels indirectly through its effects on triglyceride-rich lipoproteins and, when expressed in these tissues, it therefore appears to have antiatherogenic properties.

Hepatic lipase

HL is a member of the same triglyceride lipase gene family as LPL [26]. As its name suggests, HL is synthesized primarily in hepatocytes and is localized to the hepatic sinusoids, where it is bound to HSPGs [27]. HL hydrolyzes triglycerides and probably phospholipids in VLDL remnants or IDL, leading to more efficient uptake of these remnant particles and the generation of LDL. HL also acts on LDL to generate a small dense form of LDL. Finally, HL hydrolyzes triglycerides and probably phospholipids in the larger less-dense form of HDL, called HDL_2, thus promoting its remodeling to the smaller denser HDL_3 [28,29]. HL cooperates with other gene products, such as apoA-II, apoE, LCAT, CETP and SR-BI, in its influence on HDL metabolism. The effects of HL on HDL metabolism might be partially inhibited by apoA-II [30–32].

Data from animals and humans are consistent with the concept that HL influences HDL metabolism. Transgenic mice [33–35] and rabbits [36,37] that overexpress the gene encoding HL (*Lipc*) have decreased levels of HDL-C and smaller HDL particles (as well as reduced levels of apoB-containing lipoproteins). Overexpression of *Lipc* in the liver using adenoviral-mediated gene transfer reduced HDL-C levels by ~65% in *Lipc*-deficient mice [38], 63% in *Lcat*-transgenic mice [39] and 41% in *Apoe*-deficient mice [40]. Although the catalytic activity of HL plays a role in its effects on HDL after overexpression, expression of a catalytically inactive form of HL in mice also reduced HDL-C levels by 42% [41]. This was attributed to a bridging effect by which HL mediates the binding of HDL to the hepatocyte surface by bridging between HDL and the HSPGs on the cell surface. *In vitro*, expression of *Lipc* increased SR-BI-mediated HDL cholesterol ester uptake, an effect that was caused by both the lipolytic and the bridging functions of HL. *Lipc*-deficient female mice have levels of HDL-C elevated by ~10% [42].

In humans, high plasma HL activity is associated with reduced HDL-C levels and smaller HDL particles [19]. Conversely, genetic HL deficiency is associated with modestly elevated HDL-C levels and larger HDL particles [43,44]. A common single nucleotide polymorphism in the HL promoter has been associated with lower levels of HL activity and increased levels of HDL-C, especially HDL_2 [45–47], but this finding has not been replicated in all populations [48]. Nevertheless, genetic variation at *Lipc* is thought to be an important source of variation in HDL-C levels in the general population. The relationship between HL and atherosclerosis is complex. Overexpression of *Lipc* in mice has been reported to reduce atherosclerosis as assessed by aortic cholesterol content [33]. Conversely, knockout of *Lipc* expression in mice is associated with reduced atherosclerosis in *Apoe*-deficient mice [49,50]. HL-deficient humans have been reported to be at increased risk for atherosclerotic vascular disease [44], but also have elevated levels of atherogenic lipoproteins. Humans with coronary heart disease have been reported to have increased [51] or decreased [52] post-heparin plasma HL activity levels compared with control subjects in cross-sectional studies. The regression of coronary atherosclerosis resulting from intensive lipid-lowering therapy is associated with reduction in HL activity and favorable changes in LDL buoyancy [53]. Therefore, there might be an optimal level of HL activity with regard to atherosclerosis: whereas having too little HL could impair remnant clearance and increase cardiovascular risk, having too much reduces HDL-C levels and increases risk.

Endothelial lipase

EL is a member of the same family of triglyceride lipases as are LPL and HL. It was cloned in a

human monocyte cell line (THP-1) by differential display after oxidated-LDL treatment [54], and independently by subtractive hybridization from human umbilical vein endothelial cells undergoing tube formation to monolayer [55]. It shares 45% identity with LPL, 40% identity with HL and contains three conserved catalytic residues, ten conserved cysteine residues, a 19-residue lid, four clusters of heparin-binding regions and five potential *N*-linked glycosylation sites. EL has triglyceride lipase activity, but relative to LPL and HL has substantially greater phospholipase activity, placing it at the other end of the lipolytic spectrum from LPL [56]. Overexpression of the gene encoding human EL (*LIPG*) in the livers of mice with a recombinant adenoviral vector markedly reduced plasma concentrations of HDL-C and apoA-I [54]. Although more work is required, these results suggest that EL could play a role in HDL metabolism by hydrolyzing HDL phospholipids.

Secretory phospholipase A2 group IIA

The sPLA2 family is a group of low-molecular weight secreted phospholipases [57]. The group IIA member of this family is the best known and is frequently referred to as sPLA2-IIA or simply sPLA2. It is an acute-phase protein that exhibits phospholipase activity at the sn-2 position on the phospholipid. Plasma levels of sPLA2-IIA are increased dramatically in patients with acute inflammatory conditions, such as bacterial infections, sepsis and multiorgan failure, but are also elevated in patients with chronic inflammatory diseases [58,59]. The hydrolysis of acute-phase HDL was twice and three-times more rapid and intense than that of normal HDL [60]. Transgenic overexpression of the gene encoding human sPLA2-IIA in mice results in reduced HDL-C levels [61], reduced HDL size [62], altered HDL composition [62] and increased rate of catabolism of HDL apolipoproteins and HDL-cholesterol esters [62]. *In vitro* studies using Chinese hamster ovary cell lines transfected with the gene encoding SR-BI showed that sPLA2-IIA-modified HDL was nearly twice as efficient as a substrate for cholesteryl ester transfer [63]. Interestingly, HDL-C and apoA-I levels are decreased in both acute and chronic inflammatory states [9], but the mechanisms behind this observation are poorly understood. Upregulation of the expression of the gene encoding sPLA2-IIA in both acute and chronic inflammatory states (such as in atherosclerosis itself) could be one cause of the reduced HDL-C levels associated with inflammation.

Lecithin cholesterol acyltransferase

LCAT transfers a fatty acid from phospholipid to unesterified cholesterol, thus resulting in the generation of cholesteryl ester. Although not classically thought of as a lipase, LCAT acts as a phospholipase in that the first step of the LCAT reaction is hydrolysis of phospholipid to generate the fatty acid used for generating the cholesteryl ester [64]. LCAT is found primarily on HDL, and is responsible for generating HDL cholesteryl ester, thereby having a major influence on HDL metabolism [65]. Transgenic overexpression of *Lcat* in mice [66,67] and rabbits [68] results in substantial increases in HDL-C levels. Overexpression of human *Lcat* in human a*poA-I*-transgenic [66,67] and human *apoA-I/apoA-II*-double transgenic mice [67] leads to even greater increases in the plasma concentrations of a cholesteryl ester-enriched, large HDL. Overexpression of *Lcat* in liver with recombinant adenovirus also increases HDL-C levels in mice [69]. Transgenic overexpression of human or murine *Lcat* in wild-type cholesterol-fed mice resulted in increased apoE-containing, cholesteryl ester-enriched HDL_1 and reductions in the concentrations of pre-β HDL, [66]. The overexpression of *Lcat* delayed the catabolism of apoA-I [70] and HDL cholesteryl ester [71] in rabbits. Conversely, *Lcat*-deficient mice have markedly reduced levels of HDL-C and apoA-I [72,73].

LCAT deficiency in humans is also associated with markedly reduced HDL-C and apoA-I levels [74]. HDL metabolic studies in LCAT-deficient humans demonstrated dramatically increased catabolic rates of apoA-I and especially apoA-II [75]. The role that LCAT plays in influencing HDL-C levels in the general population is uncertain. Some studies in humans have shown a positive correlation between HDL-C levels and LCAT activity [76] or LCAT mass [77].

The relationship of LCAT to atherosclerosis is also complex. Overexpression of human *Lcat* in cholesterol-fed rabbits was associated with markedly reduced atherosclerosis [78], but this effect requires the presence of functional LDLR [79]. By contrast, transgenic overexpression of human *Lcat* in mice either resulted in increased atherosclerosis [80] or afforded no evidence of protection from it [81]. Rabbits, like humans, express a functional CETP, whereas mice lack functional CETP in plasma. When human *Lcat* was overexpressed in mice that were also transgenic for human *Cetp* expression, cholesterol-induced atherosclerosis was significantly reduced compared with non-*Lcat* transgenic mice [82], suggesting that the antiatherogenic effect of LCAT requires the presence of CETP. The impact of LCAT deficiency on atherosclerosis in mice is uncertain. In one report, aortic atherosclerosis was significantly reduced in three different mouse models with *Lcat* deficiency (*Ldlr*-deficient, *Apoe*-deficient and *Cetp*-transgenic) [73]. However, in another model, LCAT deficiency was associated with increased atherosclerosis in *Ldlr*-knockout and

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Glossary

ABCA1: ATP-binding cassette protein A1
apoA-I: apolipoprotein A-I
CETP: cholesteryl ester transfer protein
EL: endothelial lipase
FFA: free fatty acids
HDL: high-density lipoproteins
HL: hepatic lipase
HSPG: heparan sulfate proteoglycans
IDL: intermediate-density lipoproteins
LCAT: lecithin-cholesterol acyltransferase
LDL: low-density lipoproteins
LDLR: low-density lipoprotein receptor
LPL: lipoprotein lipase
PLTP: phospholipid transfer protein
sPLA2: secretory phospholipase A2
SR-BI: scavenger receptor BI
VLDL: very low-density lipoproteins

Apoe-knockout mice [83]. Interestingly, in humans, LCAT deficiency is not obviously associated with increased risk of atherosclerosis [74]. Therefore, although LCAT clearly has important effects on HDL metabolism, its relationship to atherosclerosis remains unclear.

Conclusion

HDL metabolism is complex and regulated by several factors. Remodeling of the HDL particle within the plasma compartment by secreted lipases is one crucial component of the metabolism of HDL. These lipases play a major role in determining the steady-state levels of HDL-C as well as influencing the function of HDL particles. Because of their intimate relationship with HDL metabolism and function, they are likely to have important effects on atherosclerosis in humans. Indeed, several of these lipases are viable targets for new drug development.

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